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The objective of the proposed work was to develop a range of new separation and purification media based on ligand imprinting techniques to generate ligand binding sites on silica-immobilized proteins for specifically targeted compounds. Ligand imprinting take advantage of our ability to manipulate the dynamical properties of proteins using water as a plasticizer.

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DEVELOPMENT OF NOVEL SWITCHABLE PROTEIN SURFACES

FINAL REPORT

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U.S. ARMY RESEARCH OFFICE

FUNDING DOCUMENT #DAAL03-90-G-0061
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KENT STATE UNIVERSITY

DEVELOPMENT OF NOVEL SWITCHABLE PROTEIN SURFACES

OBJECTIVE

The objective of the proposed work was to develop a range of new separation and purification media based on ligand imprinting techniques to generate ligand binding sites on silica-immobilized proteins for specifically targeted compounds. Ligand imprinting take advantage of our ability to manipulate the dynamical properties of proteins using water as a plasticizer.

APPROACH

An important component of the proposed work is to understand how the dynamical properties of proteins may be manipulated by changes in hydration and temperature. Cross-polarization, Magic Angle Spinning (CPMAS) solid state ^{13}C NMR spectroscopy and positron annihilation lifetime spectroscopy were employed to define the transition from the rigid to flexible protein state. A variety of chromatographic and spectroscopic techniques were employed to optimize the protein immobilization process and to characterize the ligand binding process.

ACCOMPLISHMENTS

The work has focused on (1) the preparation and characterization of ligand-imprinted stationary phases (LISP) using lysozyme and bovine serum albumin immobilized on an amino derivatized silica and studies of the mechanism of ligand imprinting and binding, (2) dynamic studies of the bound protein under aqueous and near aqueous conditions in order to better evaluate differences in specific binding as well as non-specific solute interaction of the altered states, and (3) understanding the effects of hydration on the dynamic properties of dry and partially hydrated proteins (i.e. in the solid state).

(1) The Preparation and Characterization of Ligand-imprinted Stationary Phases.

a. Fundamental Investigations of Ligand-imprinting and Breakthrough Measurements.

This part of the work involved a number of fundamental measurements in order to examine the original ligand-imprinting concept reported by Klibanov. The results from these experiments suggests that the mechanisms controlling the imprinting and binding process are significantly different than those envisioned previously. Further, it appears that alterations of the proteins charge sites may be a significant factor in controlling solute binding under displacement mode conditions. In addition to charge alterations, other secondary protein modifiers were used to selectively modify binding. This latter approach was studied under various conditions including the use of a number of non-aqueous solvents as well as in the dry state. In the latter instance it appears that novel gas phase adsorbents can be produced from the immobilized protein systems.

b. Elution Mode Studies of Immobilized Proteins.

This part of the work involved studies under conditions where solute-surface interactions are much weaker and hence elution mode chromatography could be carried out. A number of different eluent conditions were investigated and charge manipulation was used to dramatically alter retention of target solutes on immobilized BSA sorbents. The results from these investigations have demonstrated that secondary modifiers can be used to effectively manipulate chromatographic selectivity in a controllable and switchable fashion. In addition, the results from these studies are consistent with the breakthrough measurements discussed above and further demonstrate that ligand imprinting is different than suggested by Klibanov.

(2) Studies Under Aqueous and Near Aqueous Conditions.

In this portion of the work measurements of site specific and background interactions were carried out as a function of temperature, treatment with different organic solvents and modifiers, immobilization procedure and matrix material, and source of proteins (i.e., animal species from which the protein was derived). In most cases, the separations were performed under conditions where the proteins' indigenous binding controlled solute retention. Target solutes such as D and L-tryptophan were selected which respectively provide information about the background and site specific interactions. In many cases very subtle modifications in the eluent lead to dramatic changes in the chromatographic properties of the support and in some cases non-linear retention behaviors were observed which have been attributed to physical changes in the protein. In addition to these fundamental studies, the immobilized proteins columns have been used to make various analytical measurements (e.g., determination of the kinetic and thermodynamic parameters for the mutarotation of the α and ß-isomers of di- and tri-N-acetylchitobiose).

(3) The Effects of Hydration on the Dynamic Properties of Dry and Partially Hydrated Proteins.

a. NMR Studies of Protein Hydration.

We have completed CP-MAS ^{13}C NMR studies of the effect of hydration on the dynamics and conformation for both lysozyme and bovine serum albumin (BSA).

The results of the lysozyme work have been published in Biopolymers. Little change is observed in either the proton spin lattice relaxation times or the cross relaxation times as the protein is hydrated, which indicates that there is little change in reorientational freedom of protein groups. However, the peaks observed in the aliphatic region of the NMR spectrum display a dramatically improved resolution with increases in hydration beyond a critical hydration level of between 0.1 and 0.2 g water/g protein. This enhanced resolution is due to a decrease in the distribution of isotropic chemical shifts which indicates

that the distribution of conformational states sampled by the protein decreases with increases in hydration. The onset of this effect is associated with the addition of water to the peptide backbone and other polar groups on the protein surface. Hydration, therefore, appears to order the conformation of the protein, presumably in such a way as to promote those conformations that are required for function and to suppress others.

Results of the NMR study of BSA are in press (Biopolymers). BSA show a different response to hydration compared with lysozyme. No changes are observed in the distribution of isotropic chemical shifts on hydration, with the exception of the peak at 40 ppm, which has significant contributions from methylene carbons adjacent to sulfurs in disulfide bonds (of which there are 17 in BSA). Hydration of BSA does not lead to any significant decrease in the distribution of conformational states sampled by the protein. The dry protein adopts a broad distribution of conformational states, but interconversion among these states is not possible because the dry protein is conformationally rigid. On hydration the protein regains its flexibility but retains its conformational heterogeneity.

b. Positron Annihilation Lifetime Spectroscopy

PAL spectroscopy is a very powerful method for the characterization of defects, cavity volumes, phase transitions and conformational changes in materials. We have completed the development of the data analysis methods which now make it possible to derive continuous, model-independent positron annihilation rate probability density functions and transform these to give the corresponding cavity volume distributions.

We have completed the positron annihilation lifetime (PAL) study of cavity volumes in lysozyme as a function of the hydration at 298 K. The distribution of o-positronium (o-Ps) lifetimes shifts to longer times on hydration of the protein, while the fraction of positrons forming positronium decreases. The increase in o-Ps begins at a hydration of about 0.1 - 0.15 h (g water / g protein), the same hydration level where changes in the distribution of conformational states sampled by the protein occurs, as monitored by solid-state C-13 NMR. The increase in o-Ps lifetime appears to be due to intrinsic expansion of protein free volume as well as expansion due to exchange repulsion between the positronium and the surrounding protein which can occur in the flexible, hydrated protein but not in the rigid dry protein. Other changes in the dynamic properties of proteins occur at this hydration level, suggesting that the plasticizing action of water begins at this hydration level. The decrease in positronium yields with hydration is not fully understood but may be due to a decrease in the number of cavities available for positronium or to solvation and trapping of positrons and electrons in the hydrated system which competes with positronium formation.

The changes in positron lifetime spectra for lysozyme are remarkably similar to those recently reported for polyamides. Here the plasticizing action of water on the polymer is thought to be due to disruption of inter-chain hydrogen bonding by water, which increases the flexibility of the polymer. The increase in o-Ps lifetimes is explained by exchange-repulsion effects and expansion of free volume as the polymer passes through a hydration-dependent glass transition. In polyamides hydration decreases the glass transition

temperature. The similarity in behavior suggests that the protein at a hydration of about 0.12 h undergoes a glass transition at 298 K.

There is a great deal of evidence to suggest that fully hydrated proteins undergo a glass transition at temperature of 180 - 220 K. We were, therefore, interested to determine the hydration dependence of the protein glass transition temperature. Positron lifetimes were measured as a function of temperature at hydration levels of 0.15 and 0.19 h. Increasing hydration does indeed lead to a decrease in glass transition temperature.

c. A Model of Protein Dynamical Behavior

These results taken together with others suggest a model of protein dynamic behavior in which free volume rearrangement and the plasticizing action of water play a dominant role. The chief virtue of this model of protein dynamic behavior is its ability to provide a unifying explanation for a number of seemingly unconnected observations, i.e. hydration-induced flexibility, the 200 K dynamical transition, the dynamically distinct structural classes identified by hydrogen isotope exchange studies and the differences in the temperature dependence of X-ray crystallographic B-factors. We summarize the important conclusions below:

i. We have become increasingly convinced that the size of the population of mobile buried water and its importance to protein dynamics has been greatly underestimated. While we have no direct estimate of the size of this population, several lines of evidence suggest that it is considerably larger than the population of internal water observable by X-ray diffraction. Dry proteins are rigid and glassy but are plasticized by water which appears to enter the protein interior early in the hydration process to provide alternate mobile hydrogen bond donors and acceptors for the peptide groups and thus facilitate segmental motions and free volume expansion and rearrangement.

ii. The dynamical transitions observed at low hydration at 298 K and those observed in fully hydrated proteins at 180 - 220 K represent a common glass transition in which water acts as a plasticizer of the protein. At low hydrations the glass transition temperature is much higher than the measurement temperature and the protein is rigid. As the hydration level (plasticizer content) of the protein is increased, the glass transition temperature decreases rapidly. Once sufficient water has been added to lower the glass transition temperature below the measurement temperature, the protein becomes flexible. This occurs at a hydration of 0.07 - 0.2 h at 298 K, i.e. the glass transition temperature in this hydration range is about 298 K. With further increases in hydration the glass transition temperature continues to decrease reaching a value of 180 - 220 K at full hydration.

iii. Proteins undergo dehydration-induced conformational changes which vary in extent from protein to protein. These occur predominantly at low hydrations and may involve continuous changes in conformation in some cases leading to a broad distribution of conformational states (static disorder) in the dry protein. The extent to which conformational changes appear in the dry protein is probably dependent on where along the desorption isotherm the changes occur relative to the glass transition. These continuous

conformational changes coupled with the fact that the transition from the flexible-to-rigid state are both temperature and hydration dependent offer an explanation for certain hysteresis phenomena, particularly, the differences in conformation that are observed when proteins are dried under different conditions.

iv. Many enzymes appear to be constructed from two "functional domains" each consisting of a glassy, rigid "knot" embedded in a more mobile matrix. The knots would appear to be the most significant structural element in globular proteins and define the conformation of the functional domains in which they are located. The existence of dynamically distinct regions in globular proteins appears to be due to the fact that the knot residues can pack very efficiently without compromising the strength of their hydrogen bonding. As a consequence of the low dielectric constant in the knot, the strength of all electrostatic interactions is enhanced, hydrogen bonds are shortened and the knot region contracts. This reduces mobility and lowers the local dielectric constant, which leads to further enhancements of hydrogen bond strength and further contraction. This cooperative contraction process continues until the repulsive limit is reached. In matrices there is a tendency either to sacrifice optimum packing for good hydrogen bonding or to pack well at the expense of distorting hydrogen bonds from their optimum geometry. In addition, conformational constraints imposed on the matrices by the knots, force matrices to adopt conformations with free energies higher than their intrinsic free energy minimum (Lumry's "non-intrinsic" states). In knots, good packing and strong hydrogen bonding are cooperatively enhanced. In matrices, one tends to be sacrificed for the other. Enhancement of the strength of the knots by the cooperative contraction process occurs at the expense of the matrices which further exaggerates the difference in strength of the two regions. As a consequence of this construction only the matrices undergo the 200 K glass transition, while the knots remain glassy. Only on denaturation do the knots become flexible, but the simultaneous relaxation of the constraints enforced on the matrices by the knots now allows the matrices to adjust their conformation to minimize their free energy so that a first order denaturation transition rather than a second glass transition is observed.

If correct, the model has a number of important implications both for the manipulation of protein dynamic behavior to generate LISPs and protein-based devices, as well as for the protein folding problem, tertiary structure prediction (i.e. it is the glassy "knot" regions which define the conformation of protein functional domains) and the rational design and modification of proteins by site-directed mutagenesis.

This work has potential for CBD applications, providing a basis for developing protective ensembles for the individual soldier which can detect and react to specific chemical and biological agents in the battlefield. It also has ramifications for the civilian sector, for environmental clean-up.

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